

Effect of Added Iron on the Formation of Clostridial Inhibitors

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Inhibition of *Clostridium botulinum* by nitrite was potentiated by the addition of Fe(II) or Fe(III) to the culture medium. The effect of iron was more pronounced when nitrite was added after autoclaving.

It is well established that spores of *Clostridium sporogenes* are inhibited in an organic medium autoclaved with nitrite. This effect is considerably greater than that from nitrite added after autoclaving ("Perigo effect") (7). Pivnick and Chang (8) and Ashworth and Spencer (1) found a slight Perigo effect in canned pork luncheon meat, although efforts by van Roon (9) to extract such a factor from pork heated with nitrite were unsuccessful. Chang et al. (2) implicated a Perigo-type factor in canned luncheon meat, although Johnston et al. (6) concluded from their studies that the Perigo effect is not a significant factor in preventing outgrowth of spores of *Clostridium botulinum* in commercially produced pork luncheon meat.

The conditions necessary for producing a Perigo-type inhibition in a bacteriological medium have been investigated by a number of workers. A Perigo effect was found by Johnston and Loynes (5) in the original Perigo medium and in reinforced clostridial medium, but not in liver-veal or Wynne-fluid media. The iron present in tryptone, a component of the Perigo medium, was implicated by Grever (3) as an important factor in producing Perigo inhibitor. van Roon (9) suggested black Roussin salt ($\text{Fe}_4[\text{NO}]_7\text{S}_3$) as the Perigo inhibitor in meat products but could not implicate it in clostridial inhibition. This paper reports an observation on the effect of iron on the production of antitoxin activity in bacteriological culture media.

MATERIALS AND METHODS

Culture. A strain of *C. botulinum* type A, number B1218, was obtained from the Northern Regional Research Center, Peoria, Ill. Cultures were kept in a stock medium (8 ml in 16- by 150-mm screw-cap tubes, layered with Vaspar) at pH 6.8 containing 0.5% each of tryptone, yeast extract, beef extract, and peptone; 0.2% glucose; 0.1% sodium thioglycolate; and 0.12% K_2HPO_4 . For assay, needle inoculation was made into assay medium, which had the same composition less beef extract and peptone, followed by 20 to 24 h of incubation at 35 C. These

media were autoclaved at 15 lb (ca. 6,803.4 g) for 15 min.

Assay procedure. The assay procedure was as described by Huhtanen (4) with the addition of 0 to 20 μg of iron per ml. All tests were done in duplicate (there were no differences between duplicates). Solutions to be tested were either sterilized by Seitz filtration and added to the medium after it was autoclaved, or autoclaved with the medium. One drop of a 1:50 dilution of the culture in assay medium was added to each tube (this gave about 300 cells/ml) and the tubes were incubated for 24 h at 35 C in a controlled atmosphere incubator (National Appliance Co.). The incubator was evacuated to 20 to 27 inches (ca. 60.8 to 68.7 cm) of mercury and the vacuum was replaced with nitrogen. Growth in the tubes was evaluated visually; however, when 20 μg of iron per ml was used, formation of a black color interfered with the observation of turbidity. Growth of the organisms was then verified by observing gas evolution on plunging a hot needle into the tube. The minimum inhibitory concentration of NaNO_2 needed to inhibit the growth of the organism was calculated in micrograms per milliliter.

RESULTS AND DISCUSSION

Since Grever (3) implicated iron as a component of an anticlostridial inhibitor, it was of interest to determine the amount of iron present in the assay medium. Analysis of the medium and its components showed 0.5 μg of iron per ml, divided almost equally between yeast extract and tryptone.

The effect of adding Fe(III) to the test medium before autoclaving is shown in Table 1. The Fe(III) alone did not inhibit growth in tubes without NaNO_2 . In the absence of added iron, or with a low level (0.20 $\mu\text{g}/\text{ml}$), a Perigo effect was observed; i.e., more inhibitor was produced when sodium nitrite was autoclaved in the medium than when it was added aseptically after autoclaving. The Perigo Index, the ratio of the minimum inhibitory concentration of sodium nitrite added after autoclaving to the minimum inhibitory concentration of sodium nitrite autoclaved with the medium, was the

TABLE 1. Effect of added iron on inhibition of *C. botulinum* by sodium nitrite

Added Fe(III) ^a (μg/ml)	NaNO ₂ (MIC, μg/ml) ^b	
	Before autoclaving	After autoclaving
0	5.0	80
0.20	5.0	80
2.0	5.0	40
20	2.5	5.0

^a As FeCl₃·6H₂O, added before autoclaving. Good growth was obtained in tubes with all levels of Fe(III) without nitrite.

^b MIC, Minimum inhibitory concentration.

same, 16, at both the 0 and 0.20-μg/ml Fe(III) concentrations. However, increasing the quantity of Fe(III) increased inhibition of nitrite added after autoclaving, with a concomitant decrease in the Perigo Index from 8 (with 2.0 μg/ml) to 2 (with 20 μg/ml), respectively. Increasing the concentration of iron beyond 20 μg/ml produced a black solution totally obscuring turbidity; we did not test such media. We demonstrated therefore that in a medium containing added Fe(III) a potentiated anticrostrial effect was produced without autoclaving sodium nitrite with the medium, a condition necessary for producing the Perigo inhibition.

Fe(III) was used in the first series of experiments but, since the medium contained reducing components and growth occurred under anaerobic conditions, it was of interest to determine whether reduced iron, Fe(II), would be more effective in producing inhibition in the medium. Comparison of the inhibition produced in media containing oxidized and reduced forms of iron showed (Table 2) that Fe(II) and Fe(III), tested at 20 μg/ml, were equally effective whether they were added to the medium before or after autoclaving. There was an eightfold decrease in the Perigo Index. Under these conditions it was difficult to determine which form of iron was involved in increased inhibitory activity.

The variation in Perigo Index between the experiments in Tables 1 and 2 prompted us to determine whether these might be attributable to variations in the reducing intensity. Six reductants, added before autoclaving at 0.1% final concentration, were tested: (i) a sodium thioglycollate preparation which was yellow in color due to age; (ii) old sodium thioglycollate, but with no yellowing; (iii) newly purchased sodium thioglycollate; (iv) ascorbic acid; (v) thioglycollic acid; and (vi) cysteine-hydrochloride. All media were adjusted to pH 7.0. Resazurin at 1 μg/ml was added to the media. Visual

observations of reducing intensity (a colorless solution indicated strong reduction, whereas a pink color indicated weak reducing activity) showed compounds 3 and 5 to have the fastest reduction times, followed by 6, 1, 2, and 4. The potentiating effect of 20 μg of Fe(II) per ml on the anticrostrial activity in media with these reductants is shown in Table 3. There appeared to be little or no potentiating effect of Fe(II) when ascorbic acid was the reductant, whereas a considerable effect was obtained with the sulphhydryl compounds. The greatest potentiation occurred with thioglycollic acid and cysteine; the least (excluding ascorbic acid)

TABLE 2. Comparison of Fe(II) and Fe(III)^a on inhibition of *C. botulinum* by sodium nitrite

Determinants	NaNO ₂ added (MIC, μg/ml)	
	Before autoclaving	After autoclaving
No added iron	10.0	40
20 μg of Fe(II) per ml added:		
Before autoclaving	5.0	2.5
After autoclaving ^b	10.0	5.0
20 μg of Fe(III) per ml added:		
Before autoclaving	5.0	2.5
After autoclaving	5.0	5.0

^a Fe(II) as FeSO₄·7H₂O; Fe(III) as FeCl₃·6H₂O.

^b Autoclaved separately. Good growth was obtained with 20 μg of Fe(II) or -(III) per ml in control tubes without nitrite.

TABLE 3. Comparison of the effect of several reductants on iron potentiation of nitrite inhibition of *C. botulinum*

Reductant	NaNO ₂ added after autoclaving (MIC) ^a	
	No Fe(II) ^b	Fe(II) (20 μg/ml)
Oldest sodium thioglycollate	160	40 ^c
Next oldest thioglycollate	80	20 ^c
New sodium thioglycollate	>160 ^d	20 ^c
Ascorbic acid	160	80 ^e
Thioglycollic acid	>160	10 ^c
Cysteine-hydrochloride	80	<5 ^c

^a MIC, Minimum inhibitory concentration.

^b Added as FeSO₄·7H₂O.

^c Heavy black precipitate and color.

^d 160 μg/ml was highest level tested.

^e Black color but solutions transparent.

^f No black color or sediment. The tubes without nitrite showed good growth in all cases.

was with the two old sodium thioglycollate preparations. This experiment suggested that the differences in the Perigo Index in Tables 1 and 2 could, indeed, have been caused by different sources or ages of the thioglycollate preparations.

These studies suggest that a potent anticlostridial inhibitor can be produced without autoclaving nitrite in the medium. They indicate that iron is a limiting factor and sulfhydryl groups are probably necessary for its formation.

ACKNOWLEDGMENT

We are indebted to E. DellaMonica, of this Center, for the atomic absorption analyses for Fe.

LITERATURE CITED

1. Ashworth, J., and R. Spencer. 1972. The Perigo effect in pork. *J. Food Technol.* 7:111-124.
2. Chang, P.-C., S. M. Akhtar, T. Burke, and H. Pivnick. 1974. Effect of sodium nitrite on *Clostridium botulinum* in canned luncheon meat: evidence for a Perigo-type factor in the absence of nitrite. *Can. Inst. Food Sci. Technol.* 7:209-212.
3. Grever, A. B. G. 1973. Vorming van de Perigo factor in voedingsbodems. *Voedingsmiddelentechnologie* 4: 105.
4. Huhtanen, C. N. 1975. Some observations on a Perigo-type inhibition of *Clostridium botulinum* in a simplified medium. *J. Milk Food Technol.*, in press.
5. Johnston, M. A., and R. Loynes. 1971. Inhibition of *Clostridium botulinum* by sodium nitrite as affected by bacteriological media and meat suspensions. *Can. Inst. Food Technol. J.* 4:179-184.
6. Johnston, M. A., H. Pivnick, and J. M. Samson. 1969. Inhibition of *Clostridium botulinum* by sodium nitrite in a bacteriological medium and in meat. *Can. Inst. Food Technol. J.* 2:52-55.
7. Perigo, J. A., E. Whiting, and T. E. Bashford. 1967. Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats. *J. Food Technol.* 2:377-397.
8. Pivnick, H., and P.-C. Chang. 1973. Perigo effect in pork, p. 111-116. In B. Krol and B. J. Tinbergen (ed.), *Proceedings of the International Symposium on Nitrite in Meat Products*. Center for Agricultural Publishing and Documentation, Wageningen, The Netherlands.
9. van Roon, P. S. 1973. Inhibitors in cooked meat products, p. 117-122. In B. Krol and B. J. Tinbergen (ed.), *Proceedings of the International Symposium on Nitrite in Meat Products*. Center for Agricultural Publishing and Documentation, Wageningen, The Netherlands.